



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 16/18	A1	(11) International Publication Number: WO 96/25435 (43) International Publication Date: 22 August 1996 (22.08.96)
(21) International Application Number: PCT/US96/02491 (22) International Filing Date: 14 February 1996 (14.02.96) (30) Priority Data: 08/388,463 14 February 1995 (14.02.95) US (71) Applicant: BAYER CORPORATION [US/US]; 400 Morgan Lane, West Haven, CT 06512-4175 (US). (72) Inventors: KONIG, Gerhard; 10 Three Elms Road, Branford, CT 06405 (US). GRAHAM, Paul; 43 Hubinger Street, New Haven, CT 06511 (US). (74) Agent: CHAO, Mark; Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).	(81) Designated States: CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: MONOCLONAL ANTIBODY SPECIFIC FOR β A4 PEPTIDE (57) Abstract <p>The instant invention provides for monoclonal antibody which is specific for the βA4 peptide, and in particular the free C-terminus of βA4 "1-42" but not "1-43", and stains diffuse and fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. The instant invention further provides for antibody fragments and constructs thereof which have the same binding specificity. The instant invention also provides for methods of diagnosis, screening and therapeutics for treating unique forms of βA4 peptide, using the antibodies of the instant invention.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

Monoclonal Antibody Specific for BA4 Peptide

Field of the Invention

This application relates to Alzheimer's disease, particularly, to a monoclonal antibody specific for the BA4 peptide derived from Amyloid Precursor Protein, cells which produce such antibody, methods of generating such monoclonal antibodies, and to methods for using such antibodies in diagnostics and therapy.

Background

Alzheimer's Disease (AD) is an irreversible progressive neurodegenerative brain disorder. Over the course of several years the progression of AD leads to memory loss, dementia, and finally death. Currently, it is the fourth leading cause of death in the United States, accounting for approximately 100,000 deaths annually. Typically, AD affects primarily the elderly and is therefore, with the aging of modern society, expected to be an increasing health concern in the near future. Soon after the onset of the disease, patients require assistance around the clock. This represents a tremendous psychological as well as financial problem for our society. At present, no proven means for diagnosis, prevention, treatment, or cure of AD exist.

Neuropathologically, AD is characterized by massive neuronal cell loss in certain brain areas, and by the deposition of proteinaceous material in the brains of AD patients. These deposits are the intracellular neurofibrillary tangles and the extracellular β -amyloid plaques. The major protein component of the β -amyloid plaque is the BA4 peptide. Sequence analysis of purified β -amyloid plaque material and mass spectrometry showed that the maximum length of the BA4 peptide is 43 amino acids. Typically, however, species of the peptide can also end either at position 40 or position 42 (Miller *et al.*, 1993, *Arch. Biochem. Biophys.* 301:41-52). Similarly, at the N-terminus, a certain raggedness can be observed, leading to several different forms of

the peptide, starting mainly at position 1, 4 or 11 (Miller *et al.*, 1993).

Molecular cloning revealed that the BA4 peptide is derived from a much larger precursor protein termed the "Amyloid Precursor Protein" (APP) (Kang *et al.*, 1987, *Nature* 325:733-736) (Figure 1). Figure 1 illustrates the Amyloid Precursor Protein (APP) which is a transmembrane (T_m = membrane region) protein where the N-terminus is located extracellular and the C-terminus is located intracellular (cytoplasmic). BA4 is partially embedded into the membrane. Several alternatively spliced isoforms have been described, which undergo extensive post-translational modifications (Selkoe, 1994, *Ann. Rev. Neurosci.* 17:489-517). The BA4 sequence itself is located partially on the extracellular side and extends partially into the transmembrane region (Figure 2). Figure 2 (SEQ ID NO:3) illustrates the BA4 sequence, shown (encircled area) extending with its C-terminal end into the transmembrane region (T_m, boxed area) and the N-terminal end located in the extracellular part. Asterisks indicate the location of familial mutations in the APP gene; they are either flanking the BA4 sequence, or are centered in the middle portion of the BA4 sequence. The three major cleavage sites (α , β and γ) in APP are indicated. Release of BA4 was therefore postulated to occur through the proteolytic action of one or more proteases on the N-terminus (β -cut) and on the C-terminus (γ -cut) of the peptide (Figure 2) (Selkoe, 1994). The main event during the secretion of APP is at the α -cut (position 16/17 of BA4 "1-42"). This secreted APP molecule (α APPs) contains the first 16 amino acids of the BA4 sequence at its carboxyl end. The remaining cell-associated APP fragments (so called C-terminal fragments (CTFs)) contain the C-terminal portion of the BA4 sequence and extend to the cytoplasmic region of APP. Therefore, this proteolytic cut results in fragments which may not be processed in such a way that directly or indirectly leads to amyloidogenic fragments (non-amyloidogenic processing) (Selkoe, 1994).

Recently, it was demonstrated that cell lines which express large amounts of

APP through a stably transfected APP cDNA construct produce high picomolar to low nanomolar amounts of BA4 and release it rapidly into the medium (Shoji *et al.*, 1992, *Science* 258:126-129). It has also been found that virtually every primary cell culture and cell line releases BA4 constitutively (Busciglio *et al.*, 1993, *PNAS USA* 90:2092-2096). Additionally, healthy controls as well as Alzheimer patients have been shown to have low nanomolar amounts of BA4 in sera and CSF (Seubert *et al.*, 1992, *Nature* 359:325-327). The majority of the detected soluble BA4 species in these body fluids and conditioned media was BA4 "1-40", which is not truly reflective of the overall composition found in β -amyloid plaque depositions. The notion that the production and subsequent release of BA4 is sufficient and therefore responsible for the buildup of β -amyloid plaques in the brains of AD patients could therefore no longer be maintained; other factors must contribute to the deposition of β -amyloid plaques. One straightforward hypothesis is that acute or chronic overproduction of BA4 causes the increased amyloid load observed in AD

The finding that specific point mutations in and around the BA4 region of the APP gene are linked with certain familial Alzheimer's disease (FAD) cases showed unequivocally that the APP gene is a "disease gene" (Goate *et al.*, 1991, *Nature* 349:704-706; Murrell *et al.*, 1991, *Science* 254:97-99; Levy *et al.*, 1990, *Science* 248:1124-1126; Carter *et al.*, 1992, *Nature Genetics* 2:255-256). In families in which AD is inherited dominantly with a specific age of onset, the point mutations in the APP gene are necessary and sufficient to cause AD. Although the vast majority of Alzheimer disease cases are sporadic and probably multifactorial, these familial APP mutations can teach us a great deal about amyloidogenesis, i.e., the generation of the small BA4 peptide from the larger precursor and its subsequent deposition in β -amyloid plaques.

The double mutation at APP codon 670/671 (the "Swedish variant", at the N-terminus of BA4 in APP) causes a 5 to 8 fold higher release of BA4 in cell cultures

stably transfected with that mutated APP cDNA (Figure 2) (Citron *et al.*, 1992; Cai *et al.*, 1993). It is conceivable that this double point mutation leads to an increased turnover of APP due to increased proteolysis at the β -cut, which in turn leads to a higher level of released BA4. Increased amounts of BA4 monomers, as demonstrated by transfection studies with the "Swedish mutation", can explain the faster kinetics of BA4 aggregation to β -amyloid plaques in these families.

Another FAD mutation lies C-terminal of BA4 at position 717 ("London variant") and does not affect the level of released BA4 in tissue culture (Figure 2). It was recently demonstrated that this 717 mutation changes the "1-40/1-42" BA4 ratio (Suzuki *et al.*, 1994, *Science* 264:1336-1340). Although it is not clear at the moment how the generation of the C-terminus of BA4 occurs, since this part is embedded in the transmembrane region, it is tempting to hypothesize that the "London mutation" affects the proteolytic breakdown of APP to BA4. Possibly, this point mutation interferes with the cleavage fidelity of the responsible protease at the γ -site. BA4 1-40 exhibits among other things a dramatic difference in its solubility in aqueous solutions when compared to BA4 1-42 (Burdick *et al.*, 1992, *JBC* 267:546-554). The latter is virtually insoluble in water, whereas 1-40 is water soluble up to several mg/ml in vitro. Minor amounts of the longer 1-42 form can enhance precipitation of 1-40 in vitro dramatically. A slightly higher proportion of the longer 1-42 BA4 species would explain the early onset deposition of BA4 to β -amyloid plaques in patients with this "London mutation". The proportion of the 1-42 species to the shorter more soluble 1-40 species may also be one of the critical factors in the sporadic AD cases (i.e. cases where no genetic predisposition was identified). Monoclonal antibodies which specifically bind to the 1-42 species, are therefore useful to investigate the production and presence of BA4 species ending at amino acid position 42, and can be used as a diagnostic indicator of abnormal species present in AD.

Recent biochemical analyses with one antibody which recognizes BA4 ending at

position 40, and one antibody recognizing BA4 species extending to position 42 or further, showed that the contribution of the longer BA4 species might be critical for the onset of the disease (Suzuki *et al.*, 1994). However, the monoclonal antibody of Suzuki does not distinguish between BA4 1-42, 1-43, and longer BA4 species. This is also the case for another reported monoclonal antibody 2G9 (Yang *et al.*, 1994, *Neuro Report* 5:2117-2120). Therefore, in order to avoid this crossreactivity, antibodies which are specific for BA4 species ending at position 42 to the exclusion of the other forms would be very useful in order to avoid crossreactivity with membrane associated C-terminal APP fragments, which are typical cellular products not necessarily associated with the β amyloid plaques.

One monoclonal antibody recognizing BA4 1-42 has been described (Murphy *et al.*, 1994, *Am. J. Path.* 144:1082-1088). However, the BA4 1-43 peptide species was not used in these studies, thus it is not known what the exact specificity of this monoclonal antibody would be in response to the 1-43 peptide. Competition studies were only performed with BA4 peptides ending at position 40 ("1-40"), and position 44 ("1-44") and beyond with this antibody. Importantly, the antibody was reported to stain diffuse amyloid, fibrillar amyloid, intraneuronal and extraneuronal neurofibrillary tangles, but *not* vascular amyloid.

An in vitro biochemical diagnostic test for Alzheimer's disease in its early stages as well as a means of screening for at-risk AD individuals is not available. The current diagnosis of AD requires a detailed clinical evaluation which cannot give clear answers until significant symptoms of dementia and memory loss are manifested. In view of the research referred to above, BA4 1-42 represents a preclinical marker for AD. Thus, identifying the level of or the buildup of BA4 1-42, or other residue 42 terminating species, and how this may progress during the course of the disease, and how it is distributed in the brain, will provide valuable insights into monitoring the course of, as well as for specific diagnosis and possible treatment of AD.

It would be useful for preparing diagnostic tests, therapeutics and for monitoring assays of AD, to have a monoclonal antibody which, in contrast to the specificity of presently available antibodies (crossreactive with 1-43; reported not to stain vascular amyloid), does stain vascular amyloid and is specific for BA4 peptide ending at residue 42, and therefore extends the diagnostic capabilities of the art, i.e. one that recognizes the free C-terminus of BA4 1-42 and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. Such an antibody is the subject of the present application.

Summary of the Invention

The instant invention provides for monoclonal antibody which is specific for the BA4 peptide, and in particular, the C-terminus of BA4 "1-42" and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. In particular, the instant invention provides for monoclonal antibody which is specific for all BA4 peptides in which the C-terminus is residue 42 of the BA4 amino acid sequence. The instant invention further encompasses antibody fragments and constructs thereof which have the same binding specificity. The instant invention encompasses in particular the monoclonal antibody known as "Mab 369.2B" and is produced by the cell line "369.2B" which has been deposited under the Budapest Treaty with the American Type Culture Collection (ATCC) on January 26, 1995, and has been assigned the ascension number HB11829. The instant invention encompasses the use of the monoclonal antibody of the instant invention in diagnostic, purification, and therapeutic uses.

Thus one embodiment of the instant invention encompasses a monoclonal antibody specific for BA4 peptide ending at position 42, wherein said antibody binds to diffuse amyloid, fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. In one particular embodiment, the instant invention provides for a monoclonal antibody which is specific for the C-terminal amino acids of the BA4 1-42 peptide. In the most

preferred embodiment the instant invention encompasses a monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829. A preferred embodiment of the instant invention also encompasses a cell which is identified by the ATCC ascension number HB 11829. In a further embodiment of the instant invention, an immunologically reactive fragment of the monoclonal antibody of the instant invention which is capable of the same binding as the monoclonal antibody of the instant invention, is encompassed.

The instant invention also provides for methods of generating BA4 specific antibodies which recognize the free C-terminal residue 42. The instant invention also provides for methods for detecting the presence of BA4 peptides ending at position 42, in tissue comprising contacting a tissue sample with monoclonal antibody of the instant invention, by detecting the presence of monoclonal antibody in a selective fashion. The instant invention also provides for methods for selective purification of BA4 peptides ending at position 42, comprising contacting a sample to be purified with monoclonal antibody of the instant invention, separating the BA4 peptide from the sample to be purified, and isolating the BA4 peptide. In a further embodiment, the instant invention provides for methods for detection of BA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with monoclonal antibody of the instant invention, and detecting the presence of BA4 peptides.

Thus the instant invention also provides for methods for the prevention of aggregation of BA4 peptide by administering monoclonal antibody of the instant invention. In a preferred embodiment the monoclonal antibody is identicle to 369.2B, or is an immunologically active fragment with equivalent binding specificity thereof. The instant invention thus provides a means for detecting the presence of BA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. As well as a means for preventing the aggregation of BA4 peptide

comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. The instant invention provides a means for detecting and monitoring the level of BA4 peptide in tissue or fluid samples (e.g. blood, other body fluids, tissue sections, biopsy tissues etc.). In a preferred embodiment, the BA4 peptide being
5 detected, monitored, inhibited or purified is a BA4 peptide with a free carboxy terminal amino acid residue being residue number 42 of the BA4 peptide amino acid sequence.

All references to publications and patent documents in the prior or subsequent sections are hereby incorporated by reference in the entirety. Specific preferred
10 embodiments of the present invention will become more evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

The invention will be more completely understood from a consideration of the following detailed description, taken in conjunction with the drawings, in which:

15 Fig. 1 is a schematic showing the BA4 portion of the Amyloid Precursor Protein (APP), its location relative to the cell membrane, and the α , β and γ cleavage sites;

Fig. 2 shows the BA4 portion of APP, its position relative to the transmembrane region of a cell, and the three major cleavage sites (α , β and γ) in APP;

20 Fig. 3 is a diagram of the clone pGK003 which was used to generate the BA4 1-42 peptide;

Fig. 4A shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled BA4 in a wheat germ system;

25 Fig. 4B shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled BA4 from wheat germ system, immunoprecipitated with MAb 286.8A;

Fig. 5 is a graph showing immunoprecipitation of in vitro translated BA4 (IVT BA4) with 286.8A;

Fig. 6 is a diagram of the peptides used to generate the immune response (immunogen) and to screen the sera of mice;

Fig. 7 is a graph showing immunoprecipitation of in vitro translated BA4 vs. antibody concentration; -○- 286.8A, -△- 369.2B, -□- 369.6;

5 Fig. 8 is a graph showing the % of various BA4 sequences immunoprecipitated by MAb 369.2;

Fig. 9 is a graph showing epitope mapping of MAb 369.2 by competitive assay, with -■- being 35-42(OVA) (Ovalbumin coupled 35-42 BA4 peptide), -□- being 1-42 BA4 peptide, and -◆- being 1-40 BA4 peptide;

10 Fig. 10 is a photograph showing the binding of MAb 369.2B to vascular amyloid and other plaques with various morphologies.

Detailed Description of the Invention

15 The β -amyloid depositions in Alzheimer's disease brains are made up mainly of BA4 peptides which show both N- as well as C-terminal heterogeneity. The major C-terminal species, identified by peptide sequencing of β -amyloid purified from postmortem brain material, end either at position 40 or 42 of the BA4 peptide which is at most 43 residues long. In vitro experiments with synthetic BA4 peptides ending either at position 40 or 42 indicate profound physico-chemical differences. Previously, 20 the distribution of these two BA4 species in postmortem tissue as well as their generation in vitro could not be addressed due to the lack of specific antibodies against the carboxy end capable of distinguishing between subspecies of BA4 peptide.

Recent evidence suggests that release of BA4 is a normal event in virtually every cell culture. Typically high picomolar to low nanomolar concentrations of BA4 25 can be measured in serum and cerebral spinal fluid (Seubert *et al.*, 1993). This finding was surprising because it had been assumed that the production of BA4 is a pathological event since BA4 is massively deposited as β -amyloid plaques in the

cortical and hippocampal brain regions of Alzheimer disease patients. Detailed sequence analysis of the released BA4 from cell culture revealed that the major species end at position 40 (Selkoe, 1994). Amyloid plaques purified from postmortem brain show a slightly different picture: amyloid deposits of the congophilic amyloid angiopathy (CAA) are BA4 aggregates surrounding blood vessels and are predominantly BA4 1-40, whereas in contrast amyloid plaques cores (APC) which are present in the brain parenchyma and are not associated with blood vessels exhibit an N-terminal raggedness (starting most commonly at residues 1, 4 and 11) and end mainly either at position 40 or 42 (Glennner and Wong, 1984, *Biochem. Biophys. Res. Comm.* 120:885-890; Masters *et al.*, 1985, *PNAS USA* 82:4245-4249; Miller *et al.*, 1993). Occasionally, longer species ending at 43 or extending even further have been described (Miller *et al.*, 1993). Because the length of the hydrophobic C-terminus is critical for the ability of the peptide to self-aggregate in vitro (Burdick *et al.*, 1992; Jarrett *et al.*, 1993, *Biochem.* 32:4693-4697), it is entirely possible that the two distinct pathological aggregates, APC and CAA and other vascular β -amyloid plaques, can be explained by the differing properties of the two species 1-40 and 1-42. This could also be the case for the so called "diffuse plaques" (Selkoe, 1994) which are seen frequently in brains of aged humans and are not associated with AD, however, have been proposed to be precursors of fibrillar β -amyloid deposits. A non-fibrillar aggregation of BA4 has been suggested for these structures. It is therefore of primary importance to determine the tissue specific production of these longer BA4 species (i.e., those ending at position 42) and their pathological appearance in brains of AD patients. Recently three reports have been published where antibodies have been described, which distinguish 1-42 and 1-40 species of BA4 (Suzuki *et al.*, 1994; Murphy *et al.*, 1994; Yang *et al.*, 1994). Unlike the antibodies of the instant invention, the antibodies reported by Suzuki *et al.*, and Yang *et al.*, crossreact in a significant degree with both the 1-43 and 1-42 species of BA4 peptide. The antibody of Murphy *et al.*, while not

tested for binding with the 1-43 species of BA4 peptide, exhibits a different tissue binding pattern than the antibodies of the instant invention, and thus must recognize a different, or modified epitope from that recognized by the antibodies of the instant invention.

Positions 29 through 42 of the BA4 peptide lie entirely within the putative transmembrane region of the Amyloid Precursor Protein and are hydrophobic in nature (Miller *et al.*, 1993). Synthetic peptides to the C-terminal sequences in this region must overcome the ability of the 34-42 sequence to form an unusually stable β -structure which is virtually insoluble in water and strong denaturants (Halverson *et al.*, 1990, *Biochem.* 29:2639-2644) if they are to be used to elicit good immune responses against soluble BA4. We designed a hydrophilic spacer five residues long which would overcome those insolubility problems and also extend the presumed epitope away from the proximity of the carrier. To reduce the likelihood of cross-reactivity with the shorter but major BA4 species, 1-40, we chose a minimal peptidyl epitope of 8 residues corresponding to positions 35-42 of the BA4 sequence. The entire synthetic sequence designed in this way was coupled by a free sulphhydryl group on a N-terminal cysteine residue to KLH (keyhole limpet hemocyanin).

Successful use of spacers and hydrophilic residues in the production of anti-peptide antibodies is well documented as is the use of hydrophilic structures to bring insoluble haptens into solution for conjugation (McMillan *et al.*, 1983, *Cell* 35:859-863; Makela and Seppala, 1986, in Handbook of Experimental Immunology, Volume 1: Immunochimistry, Wier, D.M., editor. Blackwell Scientific Publications, Oxford. pp 3.1-3.13). The success of this method in producing specific antibodies may at least in part be attributed to the presence of a free charged carboxyl terminal, especially in context of a hydrophobic sequence, as terminal residues on peptide antigens give rise to significant proportions of anti-peptide antibodies (Gras-Masse *et al.*, 1985, in Synthetic Peptides in Biology and Medicine, Alitalo, K. *et al.*, editors,

Elsevier, Amsterdam, p 105). This, along with the selective and novel use of a minimal BA4 sequence used as an immunogen maximized the probability of producing an antibody which could distinguish between BA4 species ending at positions 42 with those that do not. Although peptide competition studies did not fine map the antigenic determinant, BA4 sequences other than 1-42 were not effective in inhibiting binding. The fact that 1-42 did not totally compete with ³⁵S-methionine-labelled in vitro-translated BA4 may be due either to the particular properties of the molecule itself or to the fact that the 35-42 peptide immunogen was presented in the context of a specific spacer and/or carrier, or that a 200-1000 fold excess of unlabelled peptide is not enough to quench the signal. Non-specific effects of N-terminal residues on antigenic activity are also well documented (Benjamini *et al.*, 1968, *Biochem.* 7:1261-1264).

The intriguing finding that 25-35 actually enhances the ability of 369.2B and other antibodies to bind to BA4 may be due to a peculiar interaction between the abstracted peptide and the full length BA4 sequence itself. Residues 26-33 are believed to exist as a random coil in aqueous solution (Halverson *et al.*, 1990) and may be able to interact with soluble BA4 in such a way that makes the C- terminus more accessible to the binding sites on antibodies.

The highly specific antibody of the instant invention, of which 369.2B is a particular example was raised against a synthetic BA4 peptide having residues 35-42, and does not recognize the shorter BA4 species 1-40 in solution or on a solid phase. Furthermore, both 1-40 and 1-43 were unable to absorb out the antibody when used immunohistochemically. A secondary screening method with medium capacity through-put for the screening of hybridoma supernatants using radioactively labelled in vitro translated BA4 was applied so that antibodies culled from the primary screening could be further selected for their ability to immunoprecipitate soluble BA4. This method can be easily adapted to other proteins/antibodies of interest. The resulting

MAB 369.2B represents a superior tool to investigate the role of BA4 peptides ending at position 42 in situ, postmortem tissue, transgenic animals, and the in vitro generation of BA4 peptides in established cellular BA4 production models, for diagnostic use, and for therapeutics.

5 The monoclonal antibody of the present invention represents an important tool needed to establish a diagnostic test kit. It permits one to measure/quantify the amount of the BA4 1-42 or derivatives thereof (e.g. 4-42 species, and other truncated forms with the "42" carboxy end) in human body fluids (CSF, serum, urine etc.) or tissues. It can also be used to study the distribution pattern of 1-42 or BA4 species ending in
10 residue 42, in AD brains compared to healthy controls. Its exceptional high avidity makes it a superior and novel tool for such testing. The monoclonal antibody here disclosed can also be used in biological model systems such as transfected cell cultures or animal models (transgenic mice), designed to measure and/or influence the presence and/or production of BA4 species ending in amino acid 42. These model systems
15 represent means to identify selective modulators of the production of BA4 ending in amino acid position 42 of the BA4 in biological systems. The antibodies of the instant invention provide for methods of preventing aggregation of BA4 peptide because the specificity of the antibody will allow for the specific interference with the free C-terminal residue, thereby interfering with and disrupting aggregation that may be
20 pathogenic in AD.

 Surprisingly, the antibody of the present invention differs from that of the prior art in that it stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid while being specific for the BA4 peptide free C-terminal residue 42. This
25 unique binding pattern shows that the antibody of the instant invention recognizes a different epitope from that of the prior art, and that the tissue distribution or accessibility of the BA4 peptide recognized by the antibody of the instant invention is also different. Further, the instant invention provides for monoclonal antibody which

can precipitate the BA4 peptide out of solution, which was not demonstrated by the antibodies of the prior art. Thus the instant invention provides for unique monoclonal antibodies which recognize a unique subset of BA4 species which has a distinct tissue distribution that is most likely a better diagnostic indicator than what was previously available, and a unique target for therapeutic intervention.

Thus the instant invention provides for antibodies, antibody fragments and constructs thereof which are specific for the BA4 species of peptide where the C-terminal ends at residue 42. The instant invention also provides for the use of such antibodies, binding fragments and constructs thereof in diagnostic, analytic, therapeutic, and biochemical purification methods which employ the binding specificity of the instant monoclonal antibodies and their use within pharmaceutical formulations.

The following examples will further explain the instant invention and are shown by way of illustration, and not by way of limitation. The following examples illustrate certain aspects of the above-identified methods and compositions as well as advantageous results.

Example 1: BA4 Peptide Expression System

Preparation of plasmid pGK002

General cloning and molecular biology procedures are found for example in Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning 2nd edition, Cold Spring Harbor Lab Press. Plasmid pMTI-26, which is a Bluescript KS clone containing 2.415 kb (kilobase pairs) of the APP sequence with a TAG stop codon followed by a BamHI site placed in frame by site-directed mutagenesis after the 42nd amino acid codon of the BA4 region, was modified by excising a 1.8 kb Xba I/Bgl II fragment and religating the plasmid after filling in the ends. The resulting construct, designated pGK002, places the consensus containing initiation codon of the BA4 sequence immediately downstream of the Bluescript T7 promoter.

Preparation of plasmid pGK003

Plasmid pGK003 (Fig. 3), used in all of the wheat germ in vitro translations of BA4 to be described below, was made by subcloning a 590 bp (base pair) NotI/Xho I fragment from pGK002 containing the entire human BA4 sequence with the mutagenized stop/Bam HI into a pSP64 polyA vector (Promega Corp.). In preparing this plasmid, pGK002 was digested with Not I and Xho I and the resulting 590 bp fragment was filled in with Klenow, isolated, and ligated with pSP64polyA linearized with Sma I. Figure 3 is a diagram of Clone pGK003. The open reading frame of BA4 1-42 is expressed in vitro from the bacterial SP6 promoter. The 3' untranslated (3'-UT) region of APP is shown in black.

Example 2: In Vitro Transcription and Translation of pGK003

Plasmid pGK003 was linearized with EcoRV and complete digestion was confirmed by agarose gel electrophoresis. The sample was extracted twice with phenol/chloroform, followed by two chloroform extractions and ethanol precipitation. The resulting pellet was washed once in 70% ethanol, partially dried under vacuum, and resuspended in TE at a concentration of 1 µg/µl.

In vitro transcripts using linearized templates at 30 µg/ml were prepared in 80 mM HEPES-KOH (pH 7.5) buffer containing 16 mM MgCl₂, 2 mM spermidine, 40 mM DTT, 3 mM ATP/CTP/GTP/UTP, 800 units/ml RNAsin Ribonuclease Inhibitor (Promega Corp.), 5 units/ml Yeast Inorganic pyrophosphatase (Sigma Corp.), and 1800 units/ml SP6 RNA polymerase (Promega Corp.). The reaction mixture was kept at 37°C for 4 hrs. The resultant transcript was verified by electrophoresis through a 1.2% agarose/TBE/EtBr gel with denatured samples (65°C x 10 min).

Transcripts were translated using Wheat germ extract (Sigma Corp.). Briefly, transcripts were heated (65°C x 10 min), mixed with the wheat germ extract containing KAc, RNAsin, and a methionine-minus amino acid mixture, and translated at 25°C for

1 hr in the presence of ^{35}S -labelled methionine (Amersham). Translation of a 4 kD (kilo dalton) BA4 protein was verified by SDS-PAGE using 16% Tris/Tricine gel (Novex). Gels were fixed and proteins visualized fluorographically using a commercial system, "Amplify" (Amersham).

5 The incorporation of label into in vitro translated BA4, which contains one methionine residue per molecule, was determined by gel slicing. 2 mm slices were solubilized in 1 ml of 30% hydrogen peroxide, 0.75M NH_4OH overnight at 37°C. Next a 10 ml volume of "Ready Value" scintillation cocktail (Beckman) was added and DPMs (Decay per minute) determined using a Beckman LS6000IC scintillation counter
10 in the auto DPM mode. Typical reactions produce ~250 ng BA4/ml, or ~56 nM.

 In vitro transcription followed by translation of the BA4 clone, pGK003, in a wheat germ system resulted in a single 4 kD protein product when visualized by fluorography on a 16% Tris-Tricine SDS polyacrylamide gel (Figure 4A). Figure 4A shows the results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW
15 markers. Lane 2: Low MW markers. Lane 3: In vitro translated BA4 in a wheat germ system. The identity of this 4 kD product was confirmed by immunoprecipitation with BA4 specific antibodies (Figure 4B). Figure 4B shows results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3:
20 In vitro translated BA4 from wheat germ system immunoprecipitated with MAb 286.8A. Transcription and translation of this as well as other BA4 clones in a combined reticulocyte lysate system (TnT) did not result in the same yield or purity of radioactively labelled BA4 (data not shown). This could be due to the short transcript or to the peculiar nature of the BA4 peptide itself.

 The monoclonal antibody 286.8A, which was raised against crude peptide 1-42
25 and maps to region 3-8 of BA4, was able to precipitate this protein in a concentration-dependent manner (Figure 5). Figure 5 graphs this immunoprecipitation of in vitro translated BA4 (IVT BA4). Increasing amounts of IVT BA4 were

immunoprecipitated with a fixed amount of 286.8A (7.4 µg) in 100 µl in RIPA buffer.

Example 3: Immunogen and Screening Peptide Preparation

Peptides were prepared by standard Fmoc solid-phase procedures. (see for example Gras-Masse *et al.*, 1985).

Peptide #959, a 14 residue synthetic peptide having an N-terminal cysteine attached to a hydrophilic DGDGD spacer and residues 35-42 of human BA4 (resulting complete sequence: CDGDGDMVGGVVIA (SEQ ID NO: 1)), was coupled to a maleimide-activated KLH (Keyhole Limpet Hemocyanin) carrier using the commercially available "Inject" Activated Immunogen conjugation kit (Pierce). Briefly, 2 mg of peptide were dissolved in 200 µl of conjugation buffer and allowed to react at room temperature for 2 hrs with 2 mg of reconstituted maleimide-activated KLH. The conjugate was purified by gel filtration and used as an immunogen for monoclonal antibody production using standard protocols as described in Example 4.

Peptide #958, a 14 residue synthetic peptide having an N-terminal cysteine attached to a GGGGG spacer and residues 35-42 of the human BA4, (resulting complete sequence: CGGGGGMVGGVVIA (SEQ ID NO: 2)), was coupled to ovalbumin by dissolving 2 mg of peptide in 200 µl 6M guanidine, 0.01M phosphate pH 7.0 and conjugated as above to 2 mg of a reconstituted maleimide activated ovalbumin. The purified conjugate was used in ELISA-screening of monoclonal fusion products. Antibodies screened in this way are specific for the "35-42" determinant rather than the spacer, cysteine bridge or carrier portions of the immunogen.

Figure 6: Illustrates the peptide used to generate the immunresponse (the immunogen) and the peptide used to screen the sera of mice, as well as fusions, in the enzyme immunoassay plate (EIA) are shown. BA4 sequence 35-42 was synthesized together with a spacer and a C-terminal Cysteine, which was then used to couple it covalently via maleimide bridge to a large carrier molecule. Both, the spacer and the

carrier molecule in immunogen and screening peptide are different in order to select for BA4 sequence specific antibodies.

ELISA (Enzyme Linked Immunosorbant Assay)

Biotinylation of MAb

The N-hydroxysuccinimide ester of biotin is used to biotinylate monoclonal antibody 286.8A. The integrity of the reagent is first verified by watching it's spontaneous hydrolysis in the absence of primary amines: an 0.2 mg/ml solution of NHS-LC-Biotin (Vector Labs, Burlingame, CA) in PBS is monitored at 260nm over time. An OD260 of 1.0 after approximately 2 hrs (rising from an initial OD260 ~ 0.55) indicates the expected hydrolysis.

In the biotinylation reaction a 66:1 molar ratio of Biotin to monoclonal 286.8A at neutral pH has been found to give optimal results when the biotinylated 286.8A was tested in an Elisa format. NHS-LC-Biotin 0.6 mg in H₂O at a concentration of 0.1 mg/ml is added (within 5 min of dissolving) to 1 ml (2 mg) of 286.8A in PBS. Nucleophilic attack of the NHS ester is allowed to occur at 25°C for 4 hrs after which 10 mg of glycine in 50 µl H₂O is added to stop the reaction. The reaction is then placed over a 10 ml cross-linked dextran desalting column equilibrated to PBS and 0.5 ml aliquots are collected. The first peak representing the IgG peak is pooled and stored at 4°C until used.

Elisa procedures

Corning 25801 96-well Elisa plates are coated overnight at 4°C with 100 µl monoclonal 4G8 or other capture antibody at 5 µg/ml typically in H₂O or buffer. The plate is then washed with PBS containing 1% Triton X-100 in a Dynatech Ultrawash plus. Wells are then blocked for 90 min with 300 µl PBS containing 1% Triton X-100 and 1% Elisa grade BSA (Blocking Buffer). After washing antigen or

unknown diluted in blocking buffer is added to the wells in triplicates and incubated at room temperature for 2 hrs. The plate is washed 2 times and 400 ng biotinylated 286.8A or other detecting antibody is added. After 30 min the plate is more extensively washed (2 times wash, 2 min soak, 2 times wash) and 100 µl preformed

5 Avidin-Biotin-Alkaline Phosphatase Complexes (Vector Labs) are added. The plate is washed (2 times wash, 2 min soak, 2 times wash, 5 min soak, 4 washes) and MUP substrate added at 0.06 mg/ml 1x diethanolamine buffer. Plates are read in a Millipore Cytofluor after 15 min using a 360 nm excitation filter and a 460 nm emission filter.

10 Example 4: Generation of Monoclonal Antibodies

Balb/c mice were immunized with multiple I.P. inoculations of KLH conjugated peptide #959. Splenocytes from immunized animals were fused with the mouse myeloma AG8 using standard protocols (Wunderlich et al., 1992, J. Immunol. Methods 147:1-11). Supernatants from resultant hybridomas were screened for

15 immunoreactivity to ovalbumin-coupled peptide #958 using standard Elisa protocols as described above. Hybridomas positive for the expression of immunoreactive MAb were cloned at least twice by limiting dilution and MAb isotype analysis was performed. Purified MAb IgG was prepared from ascites fluid using protein-A affinity chromatography.

20 After fusion screening showed that immunization of mice with peptide #959 conjugated to KLH and screened in a solid-phase ELISA format with peptide #958 coupled to ovalbumin resulted in six positive parental signals (identified as 369.1 through 369.6). Both peptides have amino acids 35-42 of the BA4 region, different N-terminal spacers, and a cysteine for covalent coupling to carrier proteins (Figure 6).

25 The free C-terminus with the charged carboxy group and a limited length of only 8 amino acids favors the generation of antibodies which are specifically directed against longer forms of BA4 peptides; shorter BA4 peptides ending before amino acid 42 would

thus not be recognized.

Figure 6 diagrams the structure of the immunogen (carrier-peptide) and the screening peptide (carrier-screening peptide) used.

Two of the six parental signals were ultimately not clonable. Of the remaining four, two gave immunoprecipitation/scintillation signals only a few percent above normal non-immune controls; the other two (identified as 369.6 and 369.2) showed signals of 18% and 19% respectively. Production of monoclonal antibodies from ascites fluid and subsequent immunopurification of these clones was done. Table I compares the data obtained with the IPISA for hybridoma supernatants and purified antibodies.

Table I

<u>Cell Line</u>	<u>Isotype</u>	<u>IPSA (supernatant)</u>	<u>IPSA (purified)</u>
369.1	IgG1/IgG2b	3%	N.D.
369.2	IgG1	19%	25% (with 5 µg)
369.3	IgG1	2%	N.D.
369.6	IgG2b	18%	7% (with 10 µg)

Table 1. Comparison of antibody activities in hybridoma cell lines. IPISA data represents the percent of in vitro translated BA4 which could be immunoprecipitated by either hybridoma supernatants or purified antibody.

Example 5: Immunoprecipitation/Scintillation Assay for Hybridoma Screening

To develop and screen for monoclonal antibodies which recognize the BA4 peptide in solution rather than when attached to a solid phase, an assay was developed in which immunoprecipitation of an ³⁵S-methionine-labelled in vitro-translated BA4 (IVT BA4) is measured. A standard amount of in vitro translated BA4 is allowed to form antibody/antigen complexes in a solution which can be optimized for ionic

strength, pH, and detergent composition. After the immune complexes are precipitated with Protein G (Omnisorb cells) and washed extensively, bound radioactivity is counted in a liquid scintillation counter, background is subtracted and the efficiency of precipitation calculated. This Immunoprecipitation/Scintillation assay (IPSA) allows for both the rapid identification and characterization of antibodies, and has been used to test a variety of BA4 antibodies. The assay can be applied in general to monoclonal hybridoma supernatants as well as polyclonal sera to identify antibodies which can be used for immunoprecipitations. Typically 18 IPSAs can be performed in one day. This is therefore a fast and informative secondary hybridoma screening method

Briefly, approximately 1.5×10^5 DPMs of ^{35}S -methionine-labeled in vitro-translated BA4 (IVT BA4) were added to 10 μl of a 10x immunoprecipitation buffer (150mM NaCl, 10%NP-40, 5% deoxycholic acid, 1% SDS, 500mM Tris pH8). To this, 90 μl of monoclonal cell supernatant from the monoclonal fusion of interest (our designation # 369) were added and allowed to react for 2 hrs at 4°C. Background was determined using 90 μl of supernatant of a non-reactive clone; the positive control was 90 μl of supernatant containing monoclonal antibody 286.8A which was made previously against a crude synthetic BA4(1-42) preparation. After 2 hrs, 40 μl of a 10% solution of Omnisorb cells (Calbiochem) equilibrated in 1x immunoprecipitation buffer (RIPA buffer, 150mM NaCl, 1%NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH8) were added and allowed to react for an additional 2 hrs at 4°C with rocking. The cells were pelleted by centrifugation for 5 min at 4500 g and 4°C, and washed 3x with 800 μl cold 1x immunoprecipitation buffer. Pellets were quantitatively transferred to scintillation vials and counted in a Beckman LS6000 scintillation counter in the Auto DPM mode. The percentage of BA4 immunoprecipitated was calculated.

Immunoprecipitation/Scintillation assays were performed with 1 μg of purified monoclonal antibody 369.2B in a total volume of 100 μl 1x immunoprecipitation buffer to which 5 μg of competing peptide were added. Incubations and precipitations

were as described above.

Figure 7 depicts the percentage of immunoprecipitated IVT BA4 as a function of antibody concentration for MAbs 369.2, 369.6 and MAb 286.8A. Under the conditions of this assay, 369.2 (and further subclone 369.2B) is approximately four times better than 369.6 in immunoprecipitating soluble IVT BA4, but precipitates a little less than half as much as 286.8A. Figure 7 shows the results of Immunoprecipitation of in vitro translated BA4 vs. antibody concentration (μg antibody/100 μl RIPA buffer) where;

$$\text{Percent BA4 immunoprecipitated} = \frac{(\text{dpm with MAb}) - (\text{dpm with non or preimmune control})}{(\text{total BA4 dpm/reaction})}$$

Percents with a given MAb concentration varied only a few percentage points between and within experiments.

IPSA for Monoclonal Characterization

Approximately 1.5×10^5 DPMs of ^{35}S -methionine-labeled in vitro-translated BA4 were added to various amounts of purified monoclonal antibody, either 369.2B, 369.6, or 286.8A, in a total volume of 100 μl 1x immunoprecipitation buffer, and allowed to react as described above. Immune complexes were precipitated with Omnisorb, washed, and counted as described above.

Example 6: Characterization of MAb 369.2B

To further characterize the best cell line, 369.2B, a competition immunoprecipitation/scintillation assay (Competition IPSA) was performed. In this variation 369.2B was added to an approximate 200 fold molar excess of unlabelled competitor peptide at the same time as labelled in vitro translated BA4 1-42. As expected, peptides to the human BA4 region, 1-40, 1-11, 1-28, 12-28, as well as the reverse peptide 40-1 did not compete with the ^{35}S -methionine-labeled in vitro-translated BA4 for immunoprecipitation, whereas the complete 1-42 peptide did

(Figure 8).

These results were corroborated in a solid-phase ELISA format: the absorbed ovalbumin-coupled screening peptide which contains the BA4 region 35-42, as well as the 1-42 peptide, compete whereas 1-40 did not (Figure 9). The decreased competitiveness of the 1-42 peptide compared to the ovalbumin coupled 35-42 may be due to conformational and/or solubility factors involving the antigenic determinant, or perhaps more simply to the particular stoichiometry of the conjugation (ovalbumin, a carrier with molecular weight of 45 kD compared to 4 kD for the 1-42 peptide, and having somewhere between 5-15 conjugatable maleimide groups per mole of carrier).

Figure 8 shows Immunoprecipitation Peptide competition/Scintillation Assay for epitope determination of MAb 369.2. Peptide competitor (5 μ g) was mixed with in vitro translated BA4 ($\sim 1.5 \times 10^5$ DPMs or ~ 200 pg) then immunoprecipitated with 2 μ g 369.2, where;

$$\text{Percent BA4 immunoprecipitated} = \frac{(\text{dpm's with MAb}) - (\text{dpm's with non or preimmune control})}{(\text{total BA4 dpm's/reaction})}$$

Percents with a given MAb concentration varied typically only a few percentage points between and within experiments.

Figure 9 shows epitope mapping of MAb 369.2 by competitive assay. C369.2 (50ng IgG/100 μ l) was preincubated with or without synthetic competitor peptides (22°C, 1 hr), then subjected to Elisa against plate-bound Ovalbumin-coupled 35 - 42 (200ng/well). Percent competition was calculated relative to MAb binding in the absence of competitor, i.e. where;

$$\% \text{ competitor} = \frac{(\text{signal w/o competitor}) - ((\text{signal w competitor}) - (\text{background}))}{(\text{signal w/o competitor})}$$

The solid square is 35-42(OVA) peptide conjugate; the open square is 1-42 peptide; and the solid diamond is 1-40 peptide.

From this data we conclude that monoclonal 369.2B is specific for the C-terminal end of the full length (1-42) BA4 peptide. Although the exact antigenic

determinant has not been fine mapped, it clearly involves residues beyond position 40 and, since the antibody was made to a short synthetic peptide the determinant is unlikely to involve other residues of BA4 which may be conformationally juxtaposed. Specifically, 369.2B is a very important tool in recognizing BA4 species ending at position 42.

One additional and interesting observation from the peptide competition assay is the enhanced immunoprecipitability of in vitro-translated BA4 by the decapeptide 25-35. This phenomena has also been seen in assays using one other monoclonal antibody (i.e., 286.8A) as well as one rabbit polyclonal antisera (data not shown). We also know from other experiments using varying amounts of detergent, specifically SDS, in IPSA assays with MAb 286.8A, that more BA4 can be immunoprecipitated with increasing amounts of detergent (data not shown). SDS, interestingly, has been shown to be ineffective in solubilizing BA4 aggregates, at least as shown by SDS-PAGE (Burdick et al., 1992). However it is not immediately clear why SDS should enhance the immunoprecipitability of BA4.

Example 7: Immunohistochemical Studies

We have undertaken immunohistochemistry studies with 369.2B. The staining pattern of 369.2B (1/10,000 dilution of a 22 mg/ml ascities purified antibody solution) when compared to the monoclonal antibody 286.8A which we have shown recognizes epitope 3-8 of BA4 and is human specific (data not shown) showed interesting differences. Results obtained from immunohistochemistry demonstrated that 369.2B is an excellent antibody (at 1/10,000 dilution) to specifically label amyloid plaques cores, diffuse as well as fibrillar amyloid deposits and vascular amyloid deposits (Figure 10).

Figure 10 is a photomicrograph showing β -amyloid plaques, blood vessels, and perivascular deposits of β A4 in a paraffin embedded 10 μ m thick section from the brain of a 76 year old female patient with Alzheimer's disease. Tissue sections were

pretreated with 88% formic acid (30 min), then labeled using an
avidin-biotin-peroxidase kit (Vector Laboratories, Burlington, CA) with
nickel-diaminobenzidine as the chromagen. Monoclonal antibody 369.2B labels
plaques with a variety of morphologies, including cored, perivascular, and diffuse
5 (non-amyloidotic) plaques. It also labels a subset of amyloidotic blood vessels.

Further studies also showed that BA4 1-43 peptide was not able to compete for
staining (more than a 1000 fold excess peptide), whereas BA4 1-42 completely
abolished the signal (Table 2). Again as expected, 1-40 or 40-1 did not compete for
staining. From these studies we can already conclude that this antibody is an excellent
10 tool for immunohistochemistry. As suggested by in vitro studies which show
physico-chemical differences between 1-40 and 1-42, it is possible that these two BA4
species show distinct patterns in Alzheimer brains. With the monoclonal antibody of
the instant invention, we are now able to begin addressing this question. Thus the
monoclonal antibody and methods of the instant invention are useful for diagnostic and
15 therapeutic uses in all immunological and related methodologies which can be applied
to the detection, monitoring, extraction, inhibition and modification of unique BA4
species, in the diagnosis and treatment of AD.

Table 2

		Monoclonal Antibody used for Staining	
<u>Competing peptide</u>		<u>N-terminal Mab 286.8A</u>	<u>C-terminal Mab 369.2B</u>
5	None/buffer	+++	+++
	None/DMSO	+++	+++
	Human "40-1"	+++	+++
	Human "1-16"	-	+++
	Mouse "1-16"	+++	+++
10	Human "1-40"	-	+++
	Human "1-42"	-	-
	Human "1-43"	-	+++
	Human "35-42" with spacer	+++	-

Table 2. Results from competition binding experiments and inhibition of staining, a

+++ indicates strong staining, - indicates no detectable staining.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: König, Gerhard
Graham, Paul

(ii) TITLE OF INVENTION: Monoclonal Antibody Specific for BA4
Peptide

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Allegretti & Witcoff, Ltd.

(B) STREET: 10 South Wacker Drive Suite 3000

(C) CITY: Chicago

(D) STATE: Illinois

(E) COUNTRY: USA

(F) ZIP: 60606

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McDonnell, John J

(B) REGISTRATION NUMBER: 26,949

(C) REFERENCE/DOCKET NUMBER: 95,216

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 312-715-1000

(B) TELEFAX: 312-715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Gly Asp Gly Asp Met Val Gly Gly Val Val Ile Ala
1 5 10

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Gly Gly Gly Gly Gly Met Val Gly Gly Val Val Ile Ala
1 5 10

10

15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 4..5

(D) OTHER INFORMATION: /label= Beta

/note= "Beta cleavage site in APP"

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 20..21

(D) OTHER INFORMATION: /label= Alpha

/note= "Alpha cleavage site in APP, residues 16/17
of BA4."

20

25

30

35

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 46..47

(D) OTHER INFORMATION: /label= Gamma
/note= "Gamma cleavage site in APP"

(ix) FEATURE:

(A) NAME/KEY: Peptide
(B) LOCATION: 5..47
(D) OTHER INFORMATION: /label= BA4
/note= "BA4 peptide"

(ix) FEATURE:

(A) NAME/KEY: Region
(B) LOCATION: 33..56
(D) OTHER INFORMATION: /label= Tm
/note= "Transmembrane region of APP"

(ix) FEATURE:

(A) NAME/KEY: Region
(B) LOCATION: 1..32
(D) OTHER INFORMATION: /label= Ex
/note= "N-terminal extracellular part of APP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu	Val	Lys	Met	Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val
1					5				10					15	
His	His	Gln	Lys	Leu	Val	Phe	Phe	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys
			20					25					30		
Gly	Ala	Ile	Ile	Gly	Leu	Met	Val	Gly	Gly	Val	Val	Ile	Ala	Thr	Val
			35				40					45			
Ile	Val	Ile	Thr	Leu	Val	Met	Leu	Lys	Lys	Lys					
			50				55								

We claim:

1. A monoclonal antibody that binds BA4 peptide associated with diffuse amyloid, fibrillar amyloid, neurofibrillary tangles, and vascular amyloid, wherein said BA4 peptide contains a free carboxy terminal amino acid that corresponds with amino acid 42 of the BA4 peptide amino acid sequence.

2. A monoclonal antibody of claim 1 that recognizes the C-terminal amino acid 42 of the BA4 peptide (SEQ ID NO:3).

3. A monoclonal antibody of claim 1 that is an IgG class antibody.

4. A monoclonal antibody of claim 1 that is an IgG1 or IgG2b subclass antibody.

5. A monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829.

6. An immunologically active fragment of the monoclonal antibody of claim 1.

7. An immunologically active fragment of the monoclonal antibody of claim 5.

8. A cell which is identified by the ATCC ascension number HB 11829.

9. A method of detecting the presence of BA4 peptide in tissue comprising contacting a tissue sample with monoclonal antibody of claim 1, and detecting the presence of monoclonal antibody.

10. A method of selectively purifying BA4 peptide comprising contacting a sample to be purified with the monoclonal antibody of claim 1, separating the BA4 peptide from the sample to be purified, and isolating the BA4 peptide.

5 11. A method of detecting of BA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with the monoclonal antibody of claim 1 and detecting the presence of BA4 peptide.

10 12. A method of detecting the presence of BA4 peptide in tissue comprising contacting a tissue sample with monoclonal antibody of claim 5 and detecting the presence of monoclonal antibody.

15 13. A method of selectively purifying BA4 peptide comprising contacting a sample to be purified with the monoclonal antibody of claim 5, separating the BA4 peptide from the sample to be purified, and isolating the BA4 peptide.

20 14. A method of detecting BA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with the monoclonal antibody of claim 5, and detecting the presence of BA4 peptide.

25 15. A method of preventing aggregation of BA4 peptide comprising administering the monoclonal antibody of claim 1.

16. A method of preventing aggregation of BA4 peptide comprising administering the monoclonal antibody of claim 5.

17. A means for detecting the presence of BA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 1.

5 18. A means for detecting the presence of BA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 5.

19. A means for preventing the aggregation of BA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 5.

10 20. A method of generating the antibody of claim 1, comprising immunizing a mammal with the peptide CDGDGDMVGGVVIA (SEQ ID NO:1) conjugated to a suitable immunological carrier.

15

1/10

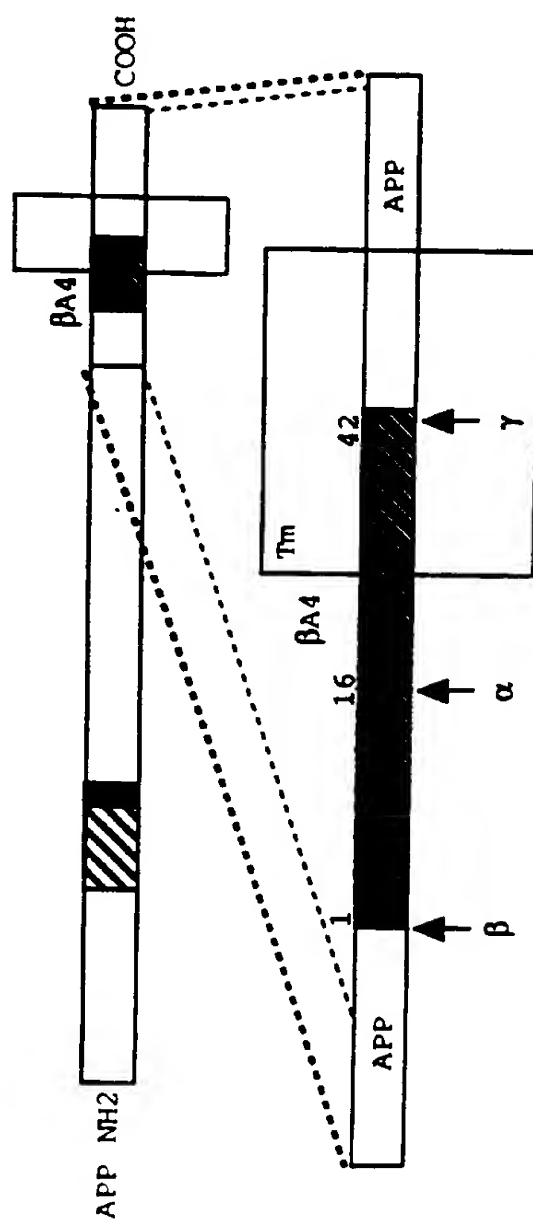


Figure 1

2/10

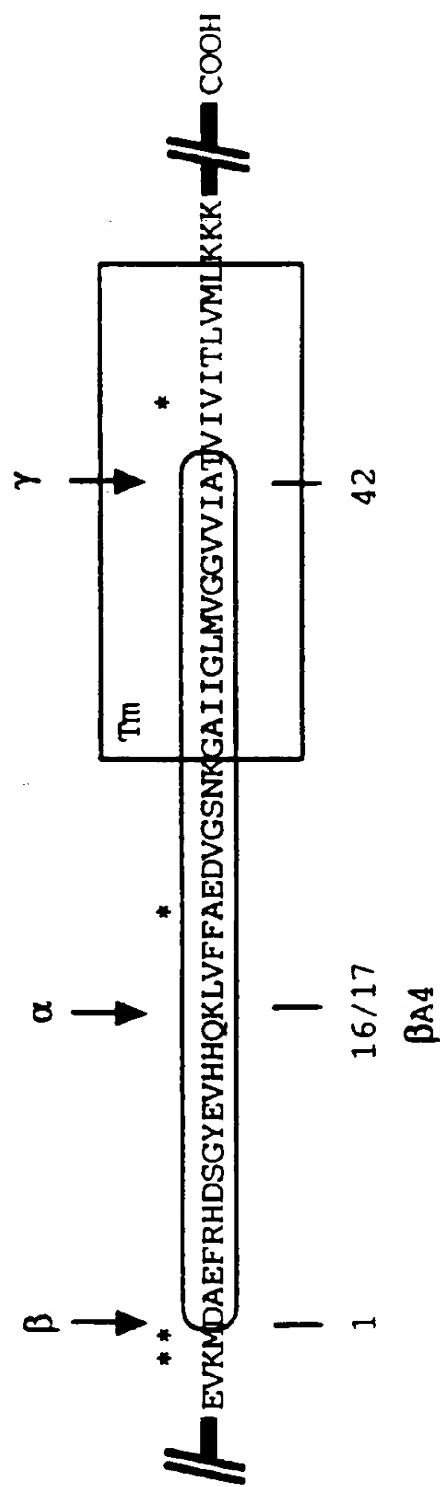


Figure 2

3/10

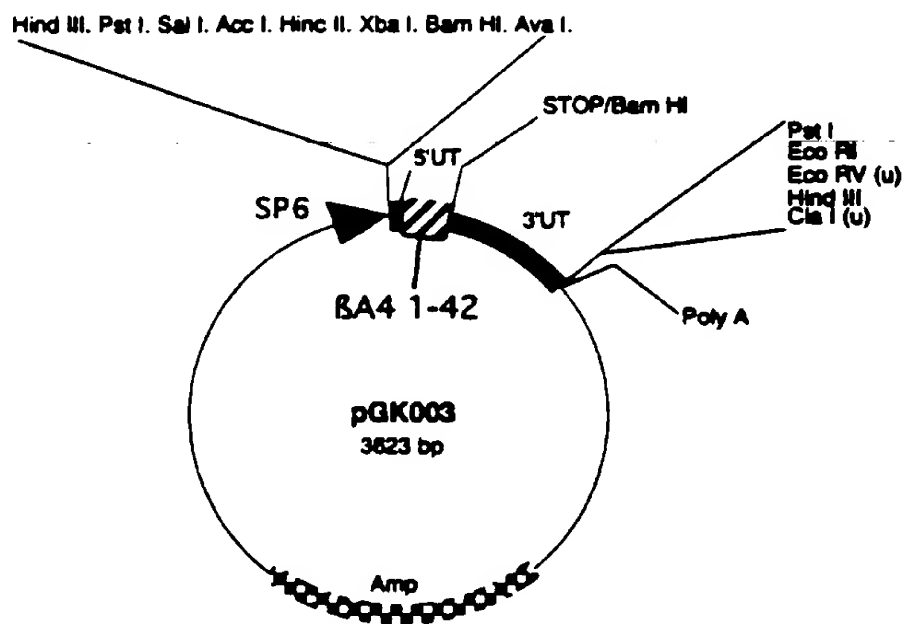


Fig. 3

4/10

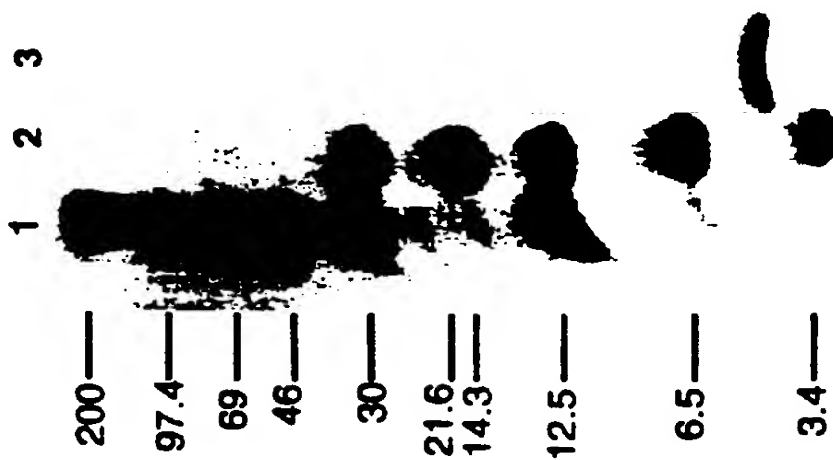


Fig 4B.

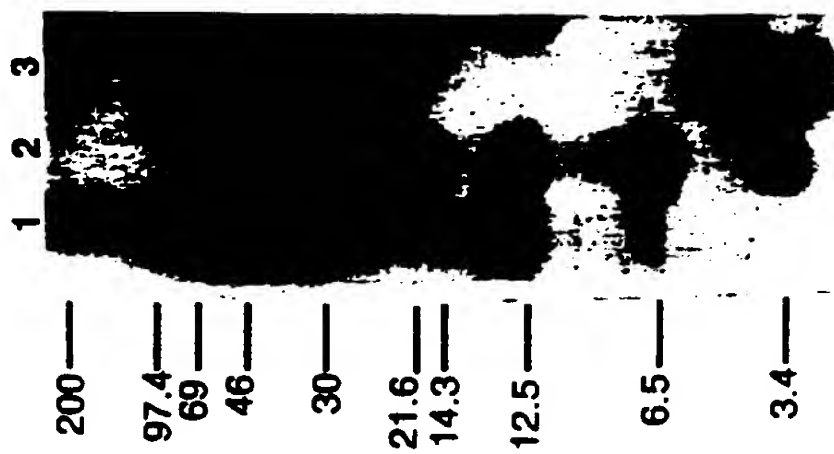


Fig. 4A.

e gel.

5/10

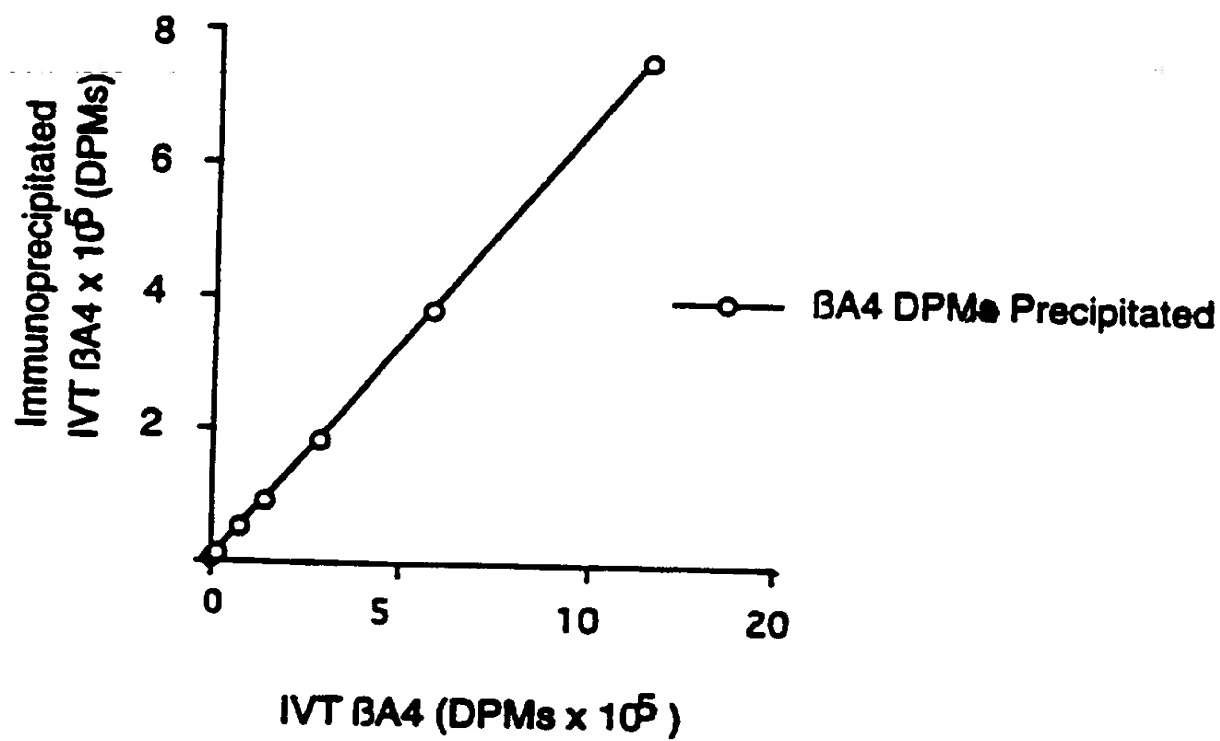


Fig. 5

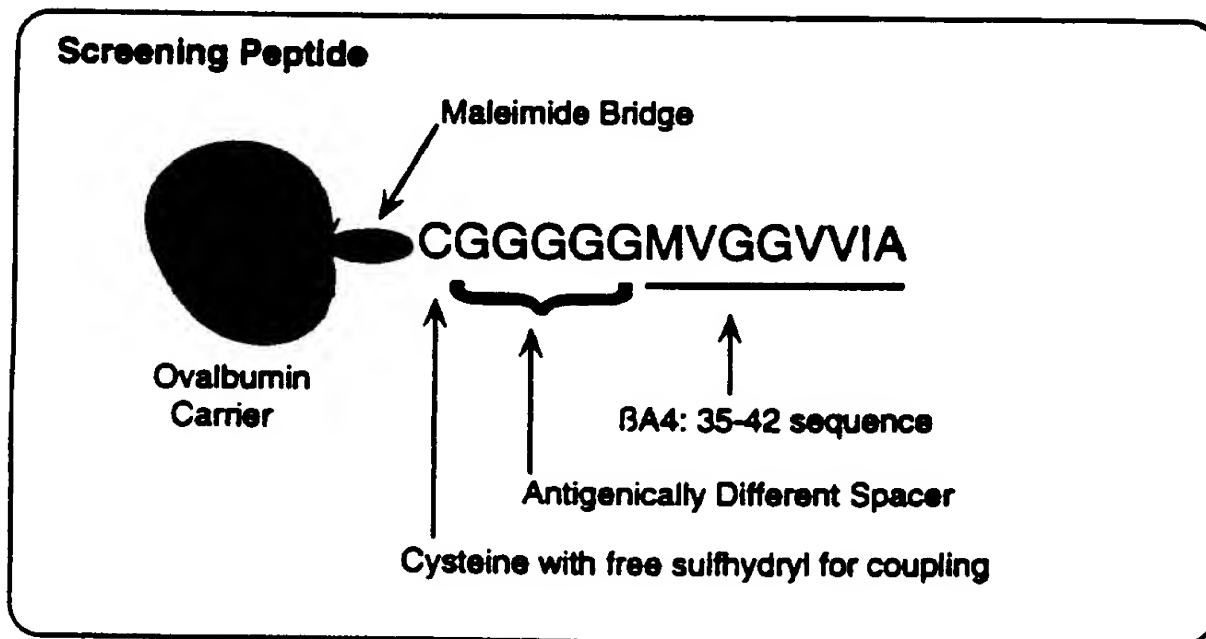
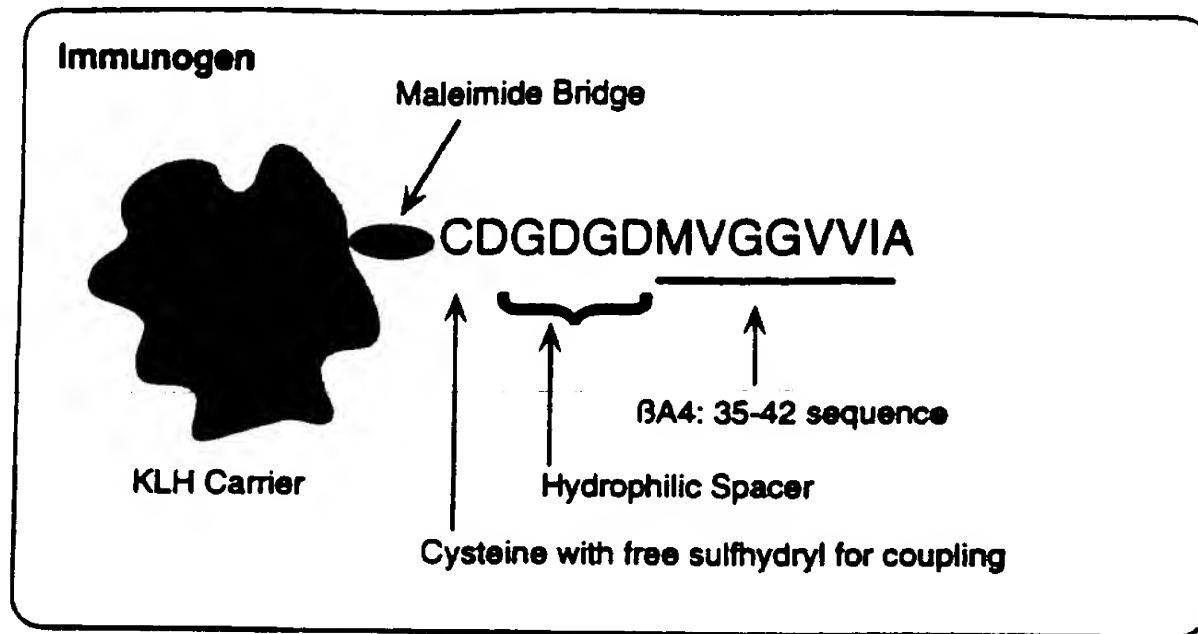


Fig 6

7/10

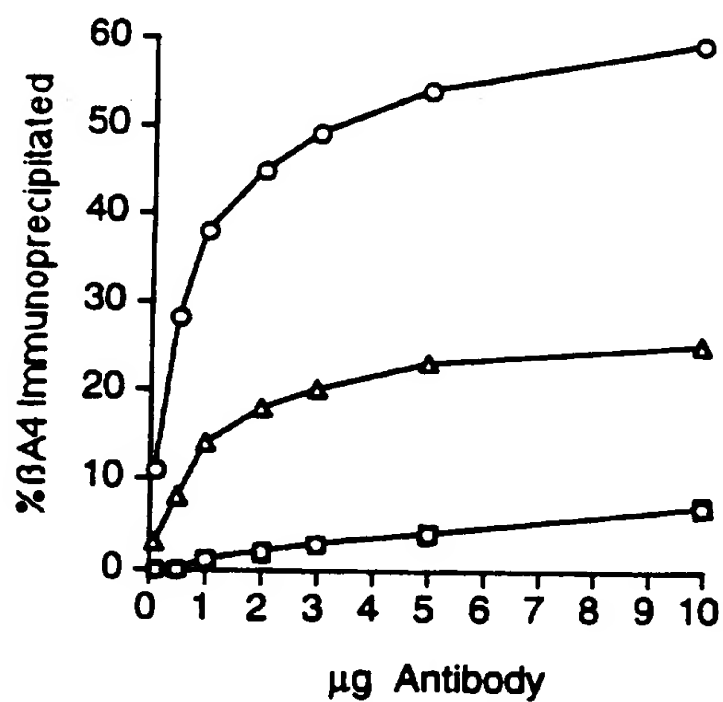


Fig. 7.

—○— 286.8A, —△— 369.2B, —□— 369.6

8/10

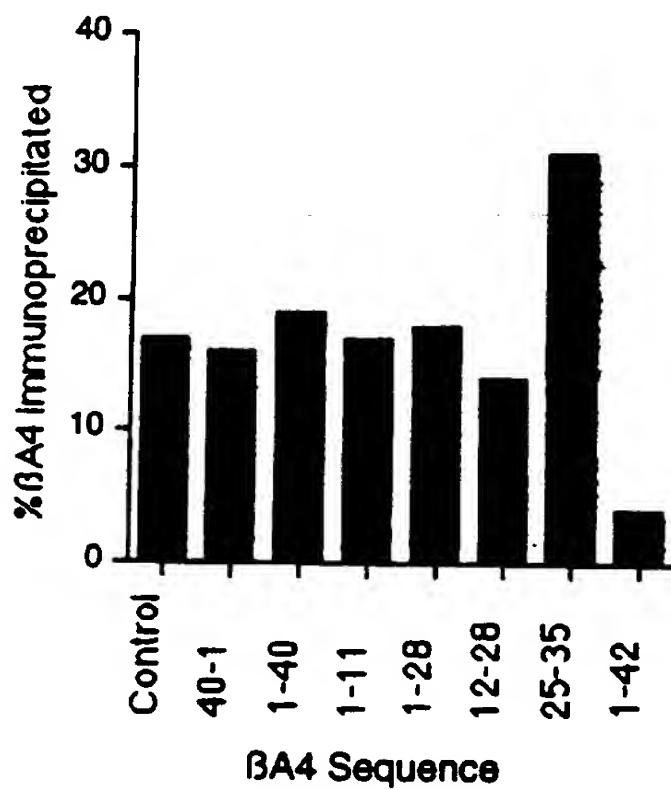


Fig. 8.

9/10

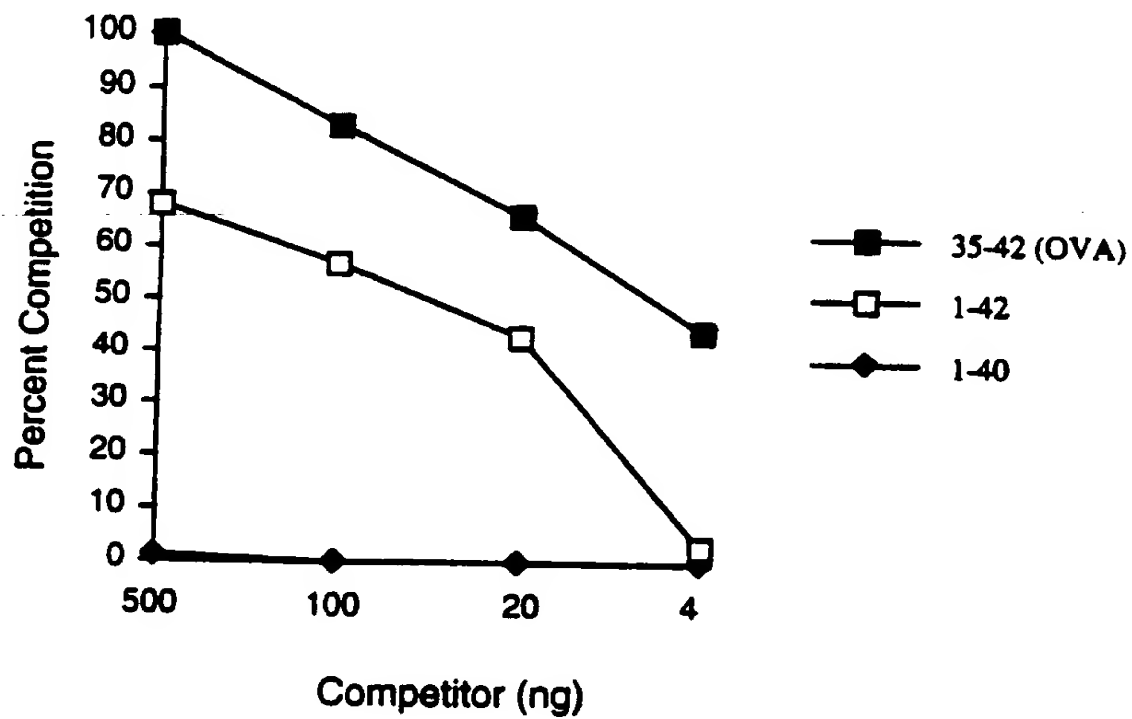


Fig. 9.

10/10

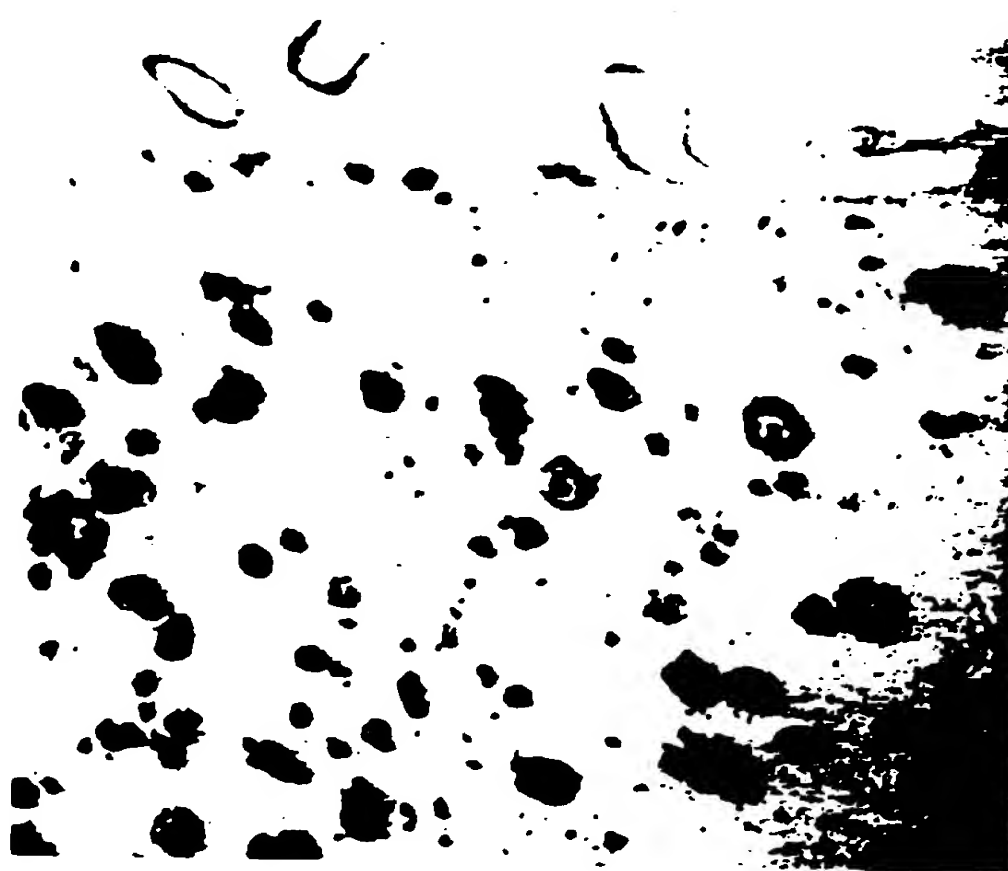


Fig. 10

International Application No
PCT/US 96/02491

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 17197 (TAKEDA CHEMICAL INDUSTRIES LTD ;SUZUKI NOBUHIRO (JP); ODAKA ASANO) 4 August 1994	1-19
Y	& EP0683234 22 NOVEMBER 1995 (SEE PAGE 4 LINE 49 -PAGE 11 LINE 25) see the whole document	20
Y	AMERICAN JOURNAL OF PATHOLOGY, vol. 144, no. 5, May 1994, pages 1082-1088, XP000573948 GREER M. MURPHY ET AL.: "DEVELOPMENT OF A MONOCLONAL ANTIBODY SPECIFIC FOR THE COOH-TERMINAL OF BETA-AMYLOID 1-42 AND ITS IMMUNOHISTOCHEMICAL REACTIVITY IN ALZHEIMER'S DISEASE AND RELATED DISORDERS." cited in the application see the whole document	20
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *A* document member of the same patent family

Date of the actual completion of the international search

28 June 1996

Date of mailing of the international search report

25.07.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/02491

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO,A,91 16628 (UNIV CALIFORNIA) 31 October 1991</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/02491

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9417197	04-08-94	EP-A- 0683234	22-11-95
WO-A-9116628	31-10-91	EP-A- 0527823	24-02-93
		US-A- 5427931	27-06-95
		US-A- 5270165	14-12-93
		US-A- 5213962	25-05-93

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15, 16, 19 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 11200

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-9 relate to a method of treatment of the human or animal body by surgery or therapy/diagnostic methods practised on the human or animal body/Rule 39.1(iv), the search has been carried out and based on the alleged effects of the compounds/compositions.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
 Information on patent family members

02/08/99

International application No.

PCT/US 99/11200

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9625435 A1	22/08/96	CA 2212887 A	22/08/96
		EP 0809656 A	03/12/97
		JP 10509736 T	22/09/98
		US 5679531 A	21/10/97
		US 5693753 A	02/12/97
		US 5786180 A	28/07/98
WO 8901343 A1	23/02/89	AT 123227 T	15/06/95
		AU 628299 B	17/09/92
		AU 2387088 A	09/03/89
		CA 1309340 A	27/10/92
		DE 3853923 D,T	16/11/95
		EP 0557270 A,B	01/09/93
		JP 3500644 T	14/02/91
		US 5004697 A	02/04/91